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Oligonucleotides Derived from 5-(1-Propynyl)-2'-*O*-Allyl-Uridine and 5-(1-Propynyl)-2'-*O*-Allyl-Cytidine: Synthesis and RNA Duplex Formation

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Abstract: The protected nucleoside analogs of 5-(1-propynyl)-2'-*O*-allyl-uridine and 5-(1-propynyl)-2'-*O*-allyl-cytidine are described. Oligonucleotides containing this modification significantly enhance double-helix formation with single-strand RNA.

The use of oligonucleotides as antisense inhibitors of gene expression[1] and probes for RNA processes[2] requires high binding affinity for RNA and resistance to nuclease degradation. 2'-*O*-alkyl oligonucleotides have been used as antisense probes for the study of pre-mRNA splicing and the structure of spliceosomes.[2] The 2'-*O*-allyl derivatives (**2**, R=H) have been shown to reduce non-specific binding to cellular components (HeLa cell nuclear extracts),[3] are very stable to nuclease degradation,[3] and the RNA duplex formed by hybridization with these probes is not a substrate for RNase H cleavage.[3,4] The C-5 propyne analogs of 2'-deoxyuridine (**3a**, pdU) and 2'-deoxycytidine (**3b**, pdC) have been shown to significantly enhance double-helix formation with single-strand RNA, relative to thymidine (**1a**) and 5-methyl-2'-deoxycytidine (**1b**).[5] In an effort to maximize binding affinity and nuclease stability of antisense oligonucleotides we have combined these sugar and heterocycle modifications. Reported herein is the synthesis of oligonucleotides derived from 5-(1-propynyl)-2'-*O*-allyl-uridine (**4a**, paU) and 5-(1-propynyl)-2'-*O*-allyl-cytidine (**4b**, paC). The results show that these oligonucleotides bind with very high affinity to single-strand RNA and, in conjunction with increased nuclease stability, offer distinct advantages as antisense probes.

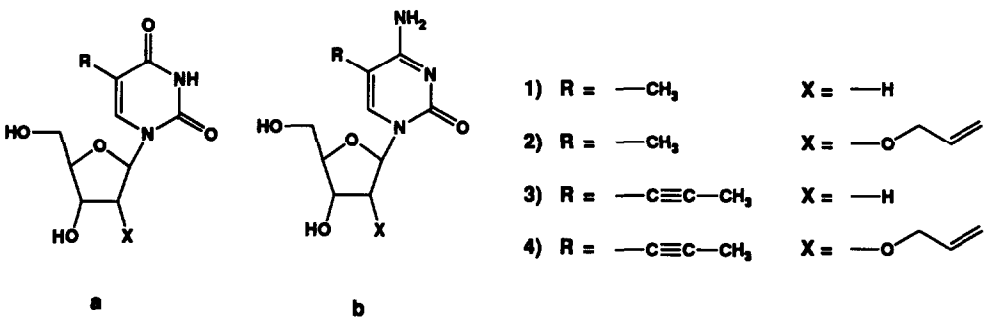
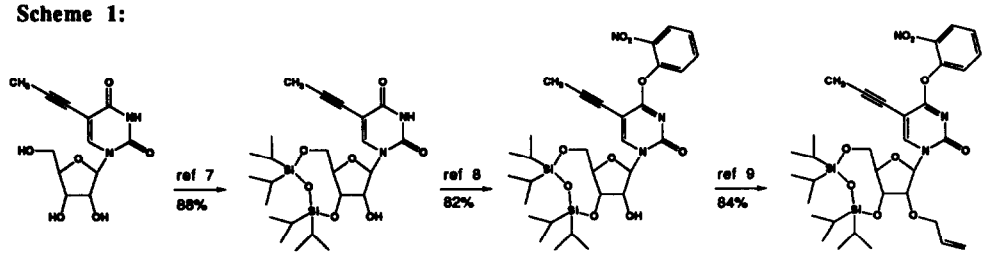


Figure 1: Structure of nucleoside analogs

Scheme 1:

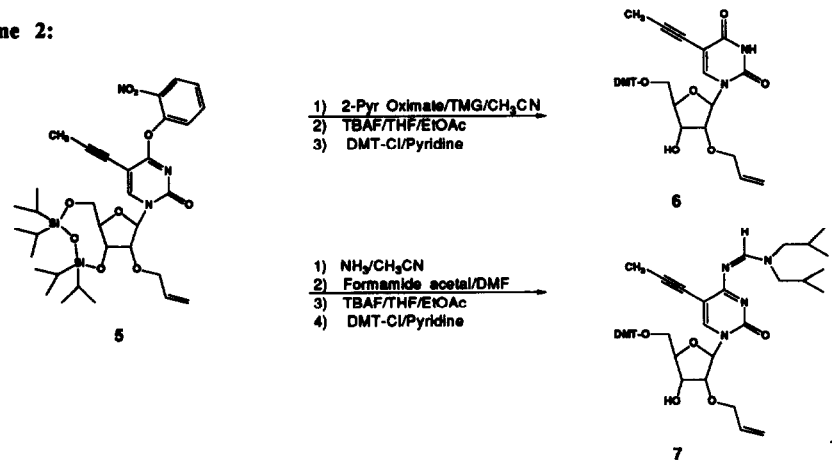


5

5-(1-Propynyl)-uridine was prepared from 5-iodouridine[6] and converted to the common precursor **5** in 61% yield (Scheme 1).[7-9] The 3 step conversion of this derivative (**5**) to the protected uridine nucleoside **6** (paU), and the 4 step conversion to the protected cytidine nucleoside **7** (paC), were accomplished without purification of the intermediates (Scheme 2, see Experimental). The N-4 of paC was protected with the diisobutyl formamidine;[10] this derivative is readily introduced and more stable than the conventional benzoyl protecting group. Phosphitylation of **6** and **7**, by standard procedures,[11] afforded the corresponding nucleoside H-phosphonates. We found that the coupling yields, utilizing the 2'-*O*-allyl nucleosides (**2** and **4**), are lower than the coupling yields with the corresponding 2'-deoxynucleosides (**1** and **3**). This problem is alleviated by increasing the final concentration of the 2'-*O*-allyl nucleoside H-phosphonate from 20 m<u>M</u> to 40 m<u>M</u> (pivaloyl chloride concentration is kept constant at 60 m<u>M</u>) in the coupling reaction.

Separate oligonucleotides[12] (Figure 2) were prepared containing each of the uridine (**2a**, **3a**, and **4a**) and cytidine (**2b**, **3b**, and **4b**) analogs shown in Figure 1. These oligonucleotides were assessed for binding to single-strand RNA (Figure 2) by thermal denaturation analysis (Tm). The Tm results show that the 2'-*O*-allyl nucleoside **2a** increases the Tm by 0.3 °C/substitution relative to the control (ΔTm = 1.5 °C) and that the 2'-*O*-allyl cytidine derivative **2b** has a more substantial effect on the Tm with an increase of 0.7 °C/substitution (ΔTm = 3.5 °C) (Table 1). This is in contrast to the results obtained with the C-5 propyne modification in which the 2'-deoxyuridine (**3a**) and 2'-deoxycytidine (**3b**) analogs have similar Tm values (70.5 °C and

Scheme 2:



5 **6** **7**

control	5' TCTCTCTCTCTTTTT 3'
uridine analogs	5' TCTCTCTCTCUUUUU 3'
cytidine analogs	5' TCTCTCTCTCTTTTT 3'
RNA Target	5' AAAAAGAGAGAGAGA 3'

Figure 2: Oligonucleotide sequences and target RNA; T is thymidine (**1a**) and C is 5-methyl-2'-deoxycytidine (**1b**), U indicates the position of the uridine analogs, C indicates the position of the cytidine analogs.

70.0 °C, respectively, Table 1). The oligonucleotides derived from the 5-(1-propynyl)-2'-*O*-allyl nucleosides (**4a** and **4b**) bind with high affinity for the target RNA (Table 1). paU (**4a**) increases the Tm by 1.7 °C/substitution (ΔTm = 8.5 °C) and paC (**4b**) increases the Tm by 2.0 °C/substitution (ΔTm = 10.0 °C) relative to the control. These data also show that the effects of these two modifications (C-5 propyne and 2'-*O*-allyl) on Tm of the duplex are additive (see ΔTm column, Table 1). The difference in Tm between paU (**4a**) and paC (**4b**) (ΔTm = +1.5 °C) is apparently a result of the 2'-*O*-allyl modification (ΔTm = +2.0 °C between **2a** and **2b**) and not a result of the C-5 propyne modification (ΔTm = -0.5 °C between **3a** and **3b**).

Table 1: Double-helix (RNA) Tm Utilizing Oligonucleotide Analogs

Uridine analog	Tm (°C)	ΔTm (°C/subst.)	Cytidine analog	Tm (°C)	ΔTm (°C/subst.)
control	63.0	–			
2a	64.5	+0.3	**2b**	66.5	+0.7
3a (pdU)	70.5	+1.5	**3b** (pdC)	70.0	+1.4
4a (paU)	71.5	+1.7	**4b** (paC)	73.0	+2.0

Tm values were determined in 140 mM KCl/5mM Na_2HPO_4/1mM $MgCl_2$ at pH = 6.6 and the final concentration of all oligodeoxynucleotides was ~2 μM. UV absorbance was monitored at 260 nm and Tm values are ±0.5 °C.

Nucleoside analogs combining the C-5 propyne pyrimidine and 2'-*O*-allyl sugar modifications have been synthesized. Oligonucleotides derived from these nucleosides have enhanced binding affinity for RNA, should resist nuclease degradation and not activate RNase H mediated cleavage of RNA. These oligonucleotide analogs will be valuable as probes for RNA processes in cellular extracts containing nucleases, and for the elucidation of the mechanism of antisense inhibition. The latter is especially important for the development of therapeutic applications of oligonucleotides.

Acknowledgements: We would like to thank Shalini Wadwani for Tm analysis and Matt Stephens for base compositional analysis.

EXPERIMENTAL:

Preparation of 5:

5 was prepared by the procedures described in references 7, 8 and 9, with the exception that desilation of the 2'-OH was carried out with methanesulfonic acid instead of p-toluenesulfonic acid (ref. 8), and **5** was purified by silica gel chromatography (EtOAc/hexane, from 1/4 to 2/3) in 61% overall yield. ^1H NMR (300.6 MHz, $CDCl_3$): δ 8.36 (s, 1H), 8.15 (d, J=8.1 Hz, 1H), 7.64-7.70 (m, 1H), 7.34-7.44 (m, 2H), 5.85-5.96 (m, 1H), 5.72 (s, 1H), 5.38 (d, J=17.1 Hz, 1H), 5.15 (d, J=10.4 Hz, 1H), 4.14-4.43 (m, 5H), 3.93-4.02 (m, 2H), 2.04 (s, 3H), 1.00-1.13 (m, 28H).

Preparation of 6:
343 mg (0.50 mmole) of **5** was dissolved into anhydrous CH_3CN (5 mL) and to this was added 2-pyridinealdoxime (67 mg, 0.55 mmole) and 1,1,3,3-tetramethylguanidine (75 µL, 0.6 mmole) at room temperature. After 18 hr the reaction mixture was diluted with EtOAc and washed with aq. citric acid (0.1 M). The aqueous layer was extracted with EtOAc, the combined organic layers washed with sat'd aq. $NaHCO_3$ (3 times), dried over Na_2SO_4 and evaporated. The residue was dissolved into EtOAc (5 mL) and to this was added 1 M TBAF/THF (1.5 mL, 1.5 mmole), the solution stirred for 1 hr and diluted with EtOAc. The solution was washed with sat'd aq. $NaHCO_3$ (2 times), the combined aqueous layer extracted with EtOAc (3 times), the combined organic phase dried over Na_2SO_4 and evaporated. The residue was evaporated from anhydrous pyridine (10 mL), dissolved into anhydrous pyridine (5 mL), and to this was added dimethoxytrityl chloride (200 mg, 0.6 mmole) and the solution stirred for 18 hr. The reaction mixture was evaporated to ~ 2 mL, diluted with CH_2Cl_2, washed with sat'd aq. $NaHCO_3$, dried over Na_2SO_4 and evaporated. Purification by silica gel chromatography (EtOAc/hexane, 1/1) yielded 197 mg (0.32 mmole, 64%) of **6**. ^1H NMR (300.6 MHz, $CDCl_3$): δ 8.92 (s, 1H), 8.03 (s, 1H), 7.19-7.46 (m, 9H), 6.84 (d, J=8.9 Hz, 4H), 5.96 (d, J=2.9 Hz, 1H), 5.87-5.93 (m, 1H), 5.25-5.36 (m, 2H), 4.36-4.47 (m, 2H), 4.22-4.28 (m, 1H), 4.11-4.15 (m, 2H), 3.79 (s, 6H), 3.46 (m, 2H), 2.72 (d, J=7.3 Hz, -OH), 1.61 (s, 3H). FAB MS: *m/z* calculated for $C_{36}H_{36}N_2O_8$ (M+) 624.2472, found 624.2476.

Preparation of 7:
343 mg (0.50 mmole) of **5** was dissolved into anhydrous CH_3CN (10 mL), and the solution transferred to a Parr Bomb, cooled to 0 °C, and saturated with NH_3. This was placed in an 80 °C bath for 24 hr (75 psi), cooled to room temperature and evaporated to dryness. The residue was evaporated from anhydrous DMF (10 mL), dissolved into anhydrous DMF (5 mL), and to this was added diisobutylformamide dimethylacetal (0.2 mL, 0.84 mmole) at room temperature. After 18 hr H_2O (25 µL) was added, the solution evaporated, dissolved into EtOAc (5 mL) and to this was added 1 M TBAF/THF (1.5 mL, 1.5 mmole). After 1 hr the reaction mixture was diluted with EtOAc, washed with sat'd aq. $NaHCO_3$, dried over Na_2SO_4 and evaporated. The residue was evaporated from anhydrous pyridine (10 mL), dissolved into anhydrous pyridine (5 mL), and to this was added dimethoxytrityl chloride (200 mg, 0.6 mmole) and the solution stirred for 5 hr. The reaction mixture was evaporated to ~ 2 mL, diluted with CH_2Cl_2, washed with sat'd aq. $NaHCO_3$, dried over Na_2SO_4 and evaporated. Purification by silica gel chromatography (EtOAc/hexane, from 2/3 to 3/2) yielded 242 mg (0.32 mmole, 64%) of **7**. ^1H NMR (300.6 MHz, $CDCl_3$): δ 8.85 (s, 1H), 8.08 (s, 1H), 7.17-7.50 (m, 9H), 6.83-6.86 (m, 4H), 5.90-6.08 (m, 2H), 5.35 (d, J=17.1 Hz, 1H), 5.22 (d, J=10.4 Hz, 1H), 4.57-4.63 (m, 1H), 4.28-4.38 (m, 2H), 4.02-4.10 (m, 2H), 3.79 (s, 6H), 3.38-3.48 (m, 4H), 3.16 (d, J=7.3 Hz, 2H), 2.65 (d, J=9.2 Hz, -OH), 2.22-2.26 (m, 1H), 1.95-2.04 (m, 1H), 1.68 (s, 3H), 0.91-0.95 (m, 12H). FAB MS: *m/z* calculated for $C_{45}H_{54}N_4O_7$ (MH+) 763.4071, found 763.4089.

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